Polypeptides for the Diagnosis and Therapy of Leishmaniasis

TECHNICAL FIELD

The present invention relates to compounds and methods for the detection of anti-leishmanial antibodies in individuals suspected of infection with the protozoan parasite of the genus *Leishmania* especially the infectious agent is an Indian strain and similar or closely related to Indian *Leishmania* strains. Further, the present invention provides a diagnostic kit consisting of the polypeptide as shown in SEQ ID NO: 5 or SEQ ID NO: 6 for the detection of anti-leishmanial antibodies in individuals where the immune responses are elicited against species of Indian strains and similar or closely related to Indian *Leishmania* strains, and also useful as a vaccine and therapeutic agent to prevent and treat leishmaniasis. The present invention further provides a diagnostic kit consisting of antibody raised against polypeptides as shown in SEQ ID NO: 5 or SEQ ID NO: 6 for detecting leishmanial antigens

BACKGROUND OF THE INVENTION

Leishmaniasis, a vector-borne parasitic disease, is caused by obligate intramacrophage protozoa. It is characterized by diversity and complexity. It presents itself with a wide range of clinical forms. However, there are mainly 4 clinical forms. The Visceral Leishmaniasis (VL), also known as *kala azar*, is the most severe form of the disease, which, if untreated, has a mortality rate of almost 100%. The Cutaneous Leishmaniasis (CL) produces skin ulcers on the exposed parts of the body, such as the face, arms and legs. The number of ulcers may vary from 1 to as many as 200 in some cases, causing serious disability and leaving the patient permanently scarred. The third form is Mucocutaneous Leishmaniasis (MCL), or *espundia.* It can lead to extensive and disfiguring destruction of mucous membranes of the nose, mouth and throat cavities and can involve even the cartilage. The cutaneous form may lead to disseminated form, known as Diffuse Cutaneous Leishmaniasis (DCL). Leishmaniasis is caused by a total of about 21 species, which are transmitted by about 30 species of *phlebotomine* sandflies [Herwaldt BL., 1999].

The leishmaniasis are presently endemic in 88 countries on five continents, Africa, Asia, Europe, North America and South America, and a total of 350 million people are at risk of infection. It is estimated that worldwide 12 million people are affected by leishmaniasis; this figure includes cases with overt disease and those with no apparent symptoms. Of the 1.5-2 million new cases estimated to occur annually,

only 600 000 are officially declared. Of the 500 000 new cases of VL, which occur annually, 90%, are in five developing countries: Bangladesh, Brazil, India, Nepal and Sudan. About 90% of all cases of MCL occur in Bolivia, Brazil and Peru and 90% of all cases of CL occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria, with 1-1.5 million new cases reported annually worldwide. The geographical distribution of leishmaniasis is limited by the distribution of the sandfly, its susceptibility to cold climates, its tendency to take blood from humans or animals only and its capacity to support the internal development of specific species of *Leishmania* [Desjeux P 2001].

Since 1993, regions that are *Leishmania*-endemic have expanded significantly, accompanied by a sharp increase in the number of recorded cases of the disease. The geographic spread is due to factors related mostly to development. These include massive rural-urban migration and agro-industrial projects that bring non-immune urban dwellers into endemic rural areas. Man-made projects with environmental impact, like dams, irrigation systems and wells, as well as deforestation, also contribute to the spread of leishmaniasis. AIDS and other immunosuppressive conditions increase the risk of *Leishmania*-infected people developing visceral illness [Desjeux P 2001, Paredes R *et al.,* 1997].

VL is primarily caused by *L. donovani* in the Indian subcontinent and Africa, *Leishmania infantum* in Mediterranean region and *Leishmania chagasi* in the new world; of these species *Leishmania chagasi* and *Leishmania infantum* are closely related. Although, all the above species cause VL they are genetically different from each other. The data obtained by Cupolillo E *et al.,* [1994] using numerical zymotaxonomy showed that *L.chagasi*, the new world visceralising species is similar to the old world *L.infantum*. The Zymodeme, serodeme, quantitative comparisons of nuclear DNA fragment patterns all indicates that *L.chagasi* and *L.infantum* are closely related and may represent the same species. Also, the study by Beverley S.M *et al.,* [1987] based on nuclear DNA restriction fragment patterns reveals that, the *L. chagasi* and *L. infantum* are similar and as closely related to each other as two random individuals from the same population and *L. donovani* is different from these two species. In another study using analysis of repetitive DNA sequence by Piarroux R *et al.,* [1995] it was observed that, amongst the leishmania causing VL, *L. donovani* isolated from foci in which human beings are the main reservoirs clustered in an independent branch and by contrast, *L. infantum* and *L. chagasi* are canine parasites that rarely infect human beings and thus are different.

A recent study by Mauricio I.L *et al.,* [1999] using three different approaches at different levels of resolution to explore the genetic information from leishmania species reveals a substantial amount of diversity within *L. donovani* complex. Further, RAPD had grouped *L. donovani* strains according to the geographical origins, specifically Indian and Kenyan, showing a substantial divergence within taxon.

Genetic diversity is not only common for *L. donovani*, even in *L. major* which causes cutaneous leishmaniasis, strains isolated from the same geographical area show minor chromosomal size polymorphisms in their molecular karyotypes whereas strains from different geographical areas show more significant differences suggesting that the genomes of species of leishmania are quite plastic and that chromosomal rearrangements occurs during the evolution of various species [Samaras N *et al.,* 1987]. Currently a WHO sponsored genome mapping project on *L. major* is underway. Although it has been argued that the genome map of one strain would be applicable to another, there is very little evidence to substantiate this claim. Indeed, it is known that differences in gene copy number and organization differ between *L. donovani*, *L. chagasi*, *L. major* and other species. Moreover, it is difficult to reconcile the great differences in clinical symptoms caused by different species with identical genotype [Ghosh S.S., *et al.,* 1998]. For these reasons, it is necessary to characterize important genes, which have potential to be a diagnostic or vaccine or therapeutic candidate from different geographical regions. The assignment of the parasite species based alone on geographic location or the site of infection is not satisfactory. Accordingly, correct diagnosis and classification of pathogenic *Leishmania* isolate is essential to determine the clinical prognosis and a species-specific therapeutic approach [Marfurt J., *et al.,* 2003]. One such potential gene studied widely across different species from different geographical region is Gp63 a glycolipid-anchored zinc protease of 63kDa size [Webb J.R., *et al.,* 1991; Steinkraus HB *et al.,* 1993; Roberts S C *et al.,* 1993].

In India, VL is a serious problem in Bihar, west Bengal and eastern Uttar Pradesh where, there is under-reporting of kala-azar (KA) and post kala-azar dermal leishmaniasis in women and children of 0-9 years of age. The recent epidemics in 1992 of VL killed more than 100,000 people in India and Sudan. Spraying of DDT helped control KA in India, however there are reports of the vector phlebotomus argentipes developing resistance. Also, lymphadenopathy, a major presenting feature in India raises the possibility of a new vector or a variant of the disease [Bora D., 1999].

The Post kala-azar dermal leishmaniasis (PKDL) is a sequel to KA in India and Sudan; the disease develops months to years after the patient recovery from KA. Cutaneous lesions characterize the disease and they demonstrate great variability, ranging from hypo-pigmented macules to erythematous papules and from nodules to plaques. As in leprosy, the wide clinical spectrum of PKDL reflects the immune response of the individual to the *leishmania* organism. Lesions may be numerous and persist for decades. Isolated parasites from the lesions are identical to those causing the original visceral disease.

The clinical and epidemiological findings in leishmaniases are not pathognomic and these can mimic with several endemic conditions such as malaria, tuberculosis, syphilis and fungal infections. Hence a laboratory diagnosis is required to confirm the clinical suspicion. The diagnostic tools used for each leishmanial syndrome viz. visceral, cutaneous, and mucocutaneous form, vary but the gold standard in each case remains the demonstration and isolation of the parasite from appropriate tissue [Singh S *et al.,* 2003].

The clinical signs and symptoms are not enough to differentiate VL from other similar conditions such as malaria, tropical splenomegaly syndrome schistosomiasis or cirrhosis with portal hypertension, African trypanosomiasis, milliary tuberculosis, brucellosis, typhoid fever, bacterial endocarditis, histoplasmosis, malnutrition, lymphoma, and leukemia. Hence other diagnostic methods are required [Herwaldt BL, 1999; Davidson RN, 1998]. Amongst these the most specific and standard technique is parasitological demonstration or isolation of the causative agent. Marrow obtained from sternal or iliac crest puncture is a much safer but a painful method. The aspirates are smeared on the glass slide and stained with Romanowsky's stain to demonstrate the amastigote forms of the parasite. However; on culture it can give positive results in up to 80% of the cases. Lymph gland puncture gives positive results in 60% of the cases. Juice is extracted from any enlarged lymph gland and subjected to both direct examination and culture to give the best chance of diagnosis [Williams, J. E, 1995; Manson-Bahr PEC, 1987]. Primary isolation of *L. donovani* is made on solid Novy-MacNeal- Nicolle (NNN) medium having 20-30% rabbit blood or liquid Schneider's insect medium supplemented with 10% v/v foetal calf serum (FCS). Other suitable growth media can also be used particularly for maintaining the subcultures of the promastigotes using FCS or other supplements including human urine [Singh S *et al.,* 2000]. Demonstration of the parasites in the spleen and liver is one of the most

accurate methods available to determine leishmanial infections. Ninety percent of the active cases show parasites in splenic and liver aspirates. The smallest needle possible, preferably, 21-gauge (0.8 mm) should be used to minimize the risk of complications such as hemorrhage of the spleen [Williams, J. E, 1995]. Part of the splenic aspirate can be used to make smears for direct microscopic examination and the rest should be cultured. Liver biopsy material is less likely to demonstrate parasites on direct examination or on culture; however histological examination will show amastigotes in Kupffer cells in the portal system.

Occasional reports of finding the *Leishmania* parasites in blood in patients of Kala-azar from Kenya and India have been published. Blood in anticoagulant is centrifuged at 2000g for 10 min and the cells from the buffy coat removed and used to prepare smears and inoculate cultures. The amastigotes can be found in and around Macrophage cells. The volume used in culture inoculation is important, 1-3 drops on NNN or Schneider's medium has given successful results [Manson-Bahr PEC, 1987].

The conventional microscopic methods are invasive and painful carrying risk of iatrogenic infections and fatal hemorrhages. Though demonstration of the amastigote form of parasite in the tissues is being used since its discovery as a parasitic disease in 1903, it is least sensitive and unable to detect occult and sub clinical infections. The sub clinical and latent form of infection has become a major concern in recent years, as these can flare up due to immune suppression such as in HIV infection and the infection can be transmitted through organ transplants. Serological diagnosis is based on the presence of specific humoral response as in cases of visceral leishmaniasis or cell mediated immune response in cases of cutaneous and mucocutaneous leishmaniasis, evoked by the immune system against the causative pathogen. There are ranges of serological methods available for the diagnosis of VL varying in accuracy and specificity. These included non-specific and specific tests. With on-going research newer better methods are continually becoming available.

The formol gel test is oldest serological test and has the advantage of being cheap and simple to perform. Serum obtained from about 5 ml of blood is mixed with one drop of 30% formaldehyde. A positive reaction is shown if the mixture solidifies and forms a white opaque precipitate within 20 minutes. A positive test cannot be detected until 3 months after infection and becomes negative 6 months after cure. The test is non-specific since it is based on detecting raised levels of IgG and IgM immunoglobulins which are also raised in other infections such as African

trypanosomiasis, malaria and schistosomiasis etc., [WHO expert committee report, 1991]. Several other tests based on this principle had been in use in past but very rarely used these days [Singh S, 1999].

There are number of specific serological tests and all have variable sensitivity and specificity for disease diagnosis. Some of these tests include indirect haemagglutination (IHA), counter current immunoelectrophoresis (CCIEP), Immunodiffusion (ID) etc. but all these tests are cumbersome and lack sensitivity and specificity and hence not commonly used. Some more commonly used ones are described below.

1. Leishmanin Skin Test (LST): Delayed hypersensitivity is an important feature of cutaneous forms of human leishmaniasis and can be measured by the leishmanin test, also known as the Montenegro reaction. Leishmanin is a killed suspension of whole (0.5-1 x 10^7/ml) or disrupted (250 μg protein/ml) promastigotes in pyrogen-free phenol saline. No cross-reactions occur with chagas disease, but some cross-reactions are found with cases of glandular tuberculosis and lepromatous leprosy. Leishmanin Skin Test is usually used as an indicator of the prevalence of cutaneous and mucocutaneous Leishmaniasis in human and animal populations and successful cure of the visceral leishmaniasis [Singh S, 1999, Sassi A, *et al.,* 1999]. During active kala-azar disease there will be no or negligible cell mediated immune response. However, the leishmanin antigen is not commercially available and no field study has been carried out in India.

2. Indirect fluorescent antibody test (IFAT): The Indirect fluorescent antibody test is one of the most sensitive tests available. The test is based on detecting antibodies, which are demonstrated in the very early stages of infection and undetectable six to nine months after cure. If the antibodies persist in low titers it is good indication of a probable relapse. Titers above 1/20 are significant and above 1/128 are diagnostic [Williams, J. E, 1995]. There is a possibility of a cross reaction with trypanosomal sera, however, this can be overcome by using *leishmania* amastigotes as the antigen instead of the promastigotes [Gari-Toussaint M, *et al.,* 1994].

3. Agglutination test: The DAT is a highly specific and sensitive test. It is cheap and simple to perform making it ideal for both field and laboratory use. The antigen is prepared from promastigotes *of L. donovani* and test can be carried out on plasma, serum, blood spots and whole blood. For long time DAT remained first line diagnostic tool in resource poor countries. The method uses whole, stained promastigotes either

as a suspension or in a freeze-dried form. The freeze-dried form is heat stable and facilitates the use of DAT in the field. However, the major disadvantage of DAT is the relative long incubation time of 18 h and the need for serial dilutions of blood or serum [Schallig HD *et al.,* 2001]. Another major disadvantage of DAT is that it has no prognostic value for evaluating the parasitological cure of the disease, as the test may remain positive for several years after cure. Recently, Schoone *et al* [2001] have developed a fast agglutination-screening test for the rapid detection (<3h) of anti-leishmania antibodies in serum samples and on blood collected on filter paper. The FAST utilizes only one serum dilution leading to qualitative results. The FAST offers the advantages of the DAT based on the freeze-dried antigen with respect to stability of the antigen, reproducibility, specificity and sensitivity.

4. Immunoblotting: Serodiagnosis using immunoblotting has been attempted and reported superior and stage specific. The various antigens expressed during the course of infection can also be documented. It also has an added advantage of permanent documentation. However, the technique is not user friendly and limited only to research laboratories [Herwaldt BL., 1999; Singh S, 1999; Schallig HD *et al.,* 2001].

5. Antigen Detection: The detection of antigen in the patient's serum is complicated by the presence of high level of antibodies, circulating immune complexes, serum amyloid, rheumatoid factor and auto antibodies all of which may mask immunologically important antigenic determinants or competitively inhibit the binding of free antigen. Antigen detection test would, in principle provide better means of diagnosis of leishmaniasis. Since antigen levels are expected to broadly correlate with the parasite load, the antigen detection may be an ideal alternative to the antibody detection in immunocompromised patients, where antibody response is very poor. Though a few reports are published, no satisfactory antigen detection system is currently available [Senaldi G *et al.,* 2001; Attar ZJ *et al.,* 2001]. Recently, a latex agglutination test (KATEX) for the detection of leishmanial antigens in the urine of patients with VL is developed. The results obtained with KATEX using samples collected from different foci of VL indicate that, the test works well regardless of the geographical origin of samples. The test had 100% specificity and sensitivity between 68-100% [Attar ZJ *et al.,* 2001]. Whether the test has applications for the detection of asymptomatic cases of VL and monitoring therapy is yet to be confirmed.

6. Enzyme linked immunosorbent assay (ELISA): The Enzyme Linked Immunosorbant Assay (ELISA) is a valuable tool in the serodiagnosis of

leishmaniasis. The test is useful for laboratory analysis or field applications. The ELISA can be performed easily and is adaptable for use with purified or defined antigen. The antigens used in the design of immunodiagnostic tests for leishmaniasis have traditionally been derived from promastigotes that have been cultivated in vitro or from recombinant proteins, alteration of the antigen used for ELISA and DAT from the whole promastigote or soluble antigens to more specific and potential recombinant leishmanial and peptide antigens have improved VL diagnosis [Senaldi G *et al.,* 2001]. Immunodiagnosis is greatly influenced by the antigen used. Several antigen molecules have recently been reported [Martin SK *et al.,* 1998; Rajasekariah GH *et al.,* 2001]. The excretory, secretary and metabolic antigens (Ld-ESM), released by *L. donovani* promastigotes into a protein-free medium were used for the serodiagnosis of VL by ELISA. The Ld-ESM has been found to be 100% specific and sensitive, the Positive Predictive Value was 99.99% and Negative Predictive Value was 95.45%. However further retrospective and prospective multisite evaluation is required to validate these findings [Schoone GJ *et al.,* 2001]. Lately, a variety of recombinant antigens have been developed, recently a gene related to the *L. major* gene B encoding a hydrophilic protein expressed on the surface of both promastigotes and amastigotes of *L. major* characterized by an amino acid repeating motif of 5.5 copies of a 14-amino acid sequence has been identified and shown to be expressed in *L. donovani.* The protein encoded by *L. donovani* gene B homologue contains up to 22 copies of a repetitive element in which 9 out of 14 residues are completely conserved between the two species. An ELISA using repetitive peptide sequence from *L. donovani* GBP and recombinant *L. donovani* GBP as solid-phase ligand was developed. However the limitations of this antigen are that it can be used for serodiagnosis of visceral leishmaniasis only in areas endemic for *L. donovani* but not for areas that are co-endemic for other *Leishmania* species and the specificity and sensitivity are not very high [Jensen AT etal 1999].

Raj *et al.,* [1999] have developed another recombinant protein rORFF of *L. infantum* origin for diagnosis of VL in India. The ORFF protein is encoded in the LD1 locus of chromosome 35 of *L. infantum*, an ELISA with this antigen proved to be sensitive with as little as 5ng of rORFF when performed with different groups of patients like confirmed VL, suspected VL, Intermittently treated endemic normal and non-endemic normal controls. Further the same patient groups were subjected to DAT using whole promastigote and ELISA using total soluble antigens. The ELISA using

rORFF was found to be more sensitive than others. Although this antigen is highly sensitive (95 to 100%) and specific (>90%) for VL, it also was found to be positive in 40% cases of confirmed CL due to *L. major* or *L. tropica*. Further the test needs to be evaluated by others and its utility for the field diagnosis is yet to be studied. In a recent study conducted in Mediterranean VL where *L. infantum* is the causative agent, ten recombinant and purified *leismania* antigens have been compared using ELISA method by Maalej *et al.,* [2003]. Of these recombinant antigens rgp63, a major surface antigen of leishmania which is not present on *Trypanosoma cruzi* or other kinetoplastids and rGBP had good performance but not very sensitive and specific for reliable diagnosis. It is suggested that the use of recombinant proteins from *L. infantum* rather than *L. major* would have yielded a better result.

A recombinant antigen developed by Burns et al., [1993] belonging to the kinesin family of motor proteins, recombinant K39 (rK39) has been shown to be specific for antibodies arising during VL caused by members of the *L. donovani* complex, which include *Leishmania chagasi* and *L. infantum*. This antigen, which is member of the kinesin family, encodes a protein with a repetitive epitope, consisting of 39 amino acid residues (K39) is highly sensitive and predictive of acute disease. The high anti-rK39 antibody titers have been demonstrated in VL patients but it shows no detectable anti-rK39 antibodies in cutaneous or mucocutaneous leishmaniasis. The antibody titers to this antigen directly correlate with active disease and have a tremendous potential as a means of monitoring chemotherapy and in predicting clinical relapse [Burns JM Jr *et al.,* 1993; Singh S *et al.,* 1995; Badaro R *et al.,* 1996; Singh S *et al.,* 2002; Maalej IA *et al.,* 2003; The United States Patent nos. 5,411,865 and 5,719,263]. In addition rK39 ELISA, has a high predictive value for detecting VL in immunocompromised persons, like AIDS patients [Houghton RL *et al.,* 1998]. This antigen is now commercially available in the form of antigen-impregnated nitrocellulose paper strips adapted for use under field conditions. The rK39 strip test has been found useful for the field diagnosis of kala-azar in India [Sundar S *et al.,* 1998] however the same had markedly less sensitivity in Sudan [Zijlstra EE *et al.,* 2001] and southern Europe. It is important to emphasize here that though the kinesin related antigen gene has been shown to be conserved in all visceralising species, but the same seems not to be case, because *L. donovani* is the causative agent of kala-azar in India as well as in Sudan, and in both the geographical regions they cause PKDL as a sequel to VL. But the observations of Zijlstra et al., [2001] that, the rK39 strip tests

are less sensitive (only upto 67%) in Sudan and even in Southern Europe (only upto 71.4%) [Jelinek T *et al.,* 1999] raise the valid doubt about the universal suitability of this antigen. One explanation often given for this variable sensitivity is that may be the antibody response elicited by different ethnic groups differs remarkably [Sundar S *et al.,* 2002]. Alternatively, it could also be possible that, the antigenic gene itself varies notably from strain to strain and also in different geographical regions or a variant of this antigen exist and evades immune elucidation as a result the disease goes undetected in few cases when the existing rK39 from *L. chagasi* is used for the diagnosis. Further, of the 500,000 new case of VL, which occurs annually worldwide, more than 90% of are reported from India, Bangladesh, Southern Sudan, and northeast Brazil [Sundar S et al., 2002].

India harbors majority of VL cases in the world, and the kinesin related antigen was not yet characterized from the Indian isolates of *Leishmania donovani.* It is also evident that, *L. chagasi* has the animal reservoir while the *L. donovani* does not. It is possible that, the gene differs significantly from the *L. chagasi* and the characterization of this gene will also explain reasons for the poor sensitivity of rK39 strip test in certain geographical regions. In an attempt to solve this problem, we have cloned and characterized the kinesin gene from different strains isolated from two individuals infected by *L. donovani* belonging to the same geographical region in India. One strain MHOM/IN/DD8 was the well characterized WHO reference strain for India isolated in 1980 and the second strain MHOM/IN/KE16/1998 is a recent clinical isolate from a 10 year old female Kala-azar patient from Muzaffarpur, Bihar, India. This clearly explains that, it is the variation at the gene level that may have caused reduced sensitivity in certain geographical regions. Had this kinesin gene not been characterized from Indian isolates, this information of variation at the gene level never would have become known to the scientific community. This is evident from the ELISA results, as the mean titers for the antigen from MHOM/IN/DD8 is considerably lower than for the one of MHOM/IN/KE16/1998 and *L. chagasi* rK 39.

The applicants did extensive search of the US patent database with different key words to study the previous work done on the K39 immunodominant repeat antigen and 230kD antigen. Discussed below are the few US patents by Reed on the subject concerned and the uniqueness of the applicant's antigen.

The United States Patent no. 5,411,865 by Reed in May 2, 1995 teaches about the method of detecting anti-leishmania parasite antibodies. The compound disclosed a

method for detecting anti-Leishmania parasite antibodies to a 230 kDa antigen present in *Leishmania chagasi* and *Leishmania donovani* which comprises obtaining a sample from an individual, contacting the sample with a recombinant K39 repeat regionantigen comprising the amino acid sequence as shown in SEQ ID NO: 3, and detecting the presence of anti-*Leishmania* parasite antibodies in the sample which bind to the recombinant K39 repeat regionantigen.

The United States Patent no. 5,719,263 by Reed in February 17, 1998 teaches about the 230Kd antigen present in Leishmania species. The compound disclosed is an isolated 230 kD antigen that is present in *Leishmania chagasi* and *Leishmania donovani*, and isolated polypeptides comprising one or a plurality of K39 repeat antigens. Also disclosed are DNAs encoding the 230 kD antigen and the K39 repeat antigen, and vaccine compositions comprising the antigens.

The above disclosed 230kDa antigen and the isolated polypeptide comprising the K39 repeats are reported to be not sensitive in certain geographical areas where VL is highly endemic and caused by *L. donovani*. However, it should be noted that, the K39 repeats has been characterized only from *L. chagasi* and not from *L. donovani*. The major burden in leishmaniasis throughout the world is caused by *L. donovani*. It is also evident from the reports that, the two species are genetically different. Hence, the applicants cloned and characterized K39 repeat immunodominant region from the Indian isolates of *Leishmania donovani*, which contributes significantly to the global VL burden.

The applicant's present invention discloses a 29kDa and 26kDa repeat antigen, which is present in *leishmania* species. The compounds disclosed are an isolated 29kD and 26kD antigens, which are characterized from the Indian isolates of *Leishmania donovani*. The reported antigens are entirely different from the *Leishmania chagasi* 230kDa antigen. The antigen varies significantly, more than 60% in its predicted amino acid sequence to that of K39 repeat antigen from *L. chagasi*. Also disclosed are DNA encoding the 29kD and 26kD antigen and therapeutic and vaccine compositions comprising the antigens.

The United States Patent no.5, 912,166 by Reed, *et al.*, in June 15, 1999 teaches about compounds and methods for diagnosis of leishmaniasis infection. The compounds provided include polypeptides that contain at least an epitope of the *Leishmania chagasi* acidic ribosomal antigen LcP0, or a variant thereof. Such compounds are useful in a variety of immunoassays for detecting Leishmania infection

and for identifying individuals with asymptomatic infections that are likely to progress to acute visceral leishmaniasis. The polypeptide compounds are further useful in vaccines and pharmaceutical compositions for preventing leishmaniasis.

However, the applicant's present invention does not deal with acidic ribosomal antigen LcPO.

The United States Patent No. 6,638,517 by Reed, *et al.,* in October 28, 2003, *Leishmania* antigens for use in the therapy and diagnosis of leishmaniasis teaches compositions and methods for preventing, treating and detecting leishmaniasis and stimulating immune responses in patients. The compounds provided include polypeptides that contain an immunogenic portion of one or more *Leishmania* antigens, or a variant thereof. The patent also discloses vaccines and pharmaceutical compositions comprising such polypeptides, or polynucleotides encoding such polypeptides, are also provided and may be used, for example, for the prevention and therapy of leishmaniasis, as well as for the detection of *Leishmania* infection.

The compounds provided in the above patent utilize a sequence of *L. major* origin and a fusion construct from multiple leishmania antigens. These compounds are in no way similar to the applicant's antigen

The kinesin related antigen gene has been cloned and characterized from *L. chagasi* an South American visceralising species by Burns *et al.,* [1993], this led to the development of rK39 antigen, a recombinant protein with 39 amino acid tandem repeats. This antigen from a new world visceralising species *L. chagasi* is reported not sensitive in some highly endemic regions for VL, Sudan and Southern Europe as it is on some other geographical areas, India, Bangladesh, Nepal etc., [Sundar *et al.,* 2002] India carries the majority of the VL population in the global leishmaniasis burden. So, it is very important to have precise and well characterized tools for the diagnosis and other control measures. The applicants have characterized the kinesin related antigen at the sequence level to study, whether the sequence is similar to that of *L.chagasi* which is well characterized and to rule out the possibility of some VL and PKDL cases going undetected in India as those reported in Sudan. This study will also through light on the reasons for the varied response among the ethnic groups, which harbor this disease to the rK39 antigen of Burns *et al.,* [1993]. In the present invention we found that, the sequence of our kinesin antigen significantly differs from that of the published reports of *L. chagasi* from which the rK39 of Burns *et al.,* was derived. This invention

also provides a method for detection of anti-leishmanial antibodies using the antigens derived from the Indian isolates of *L. donovani.*

The study was important to analyze the response to the antigen in the various ethnic groups in India. It is possible that the antibody levels in the diseased people of India may be due to either the strains being different or it is possible that the various ethnic groups respond to the *Leishmania* antigen in different manner.

OBJECTS OF THE INVENTION

The main object of this invention is to isolate, characterize and use the immunodominant region of the kinesin related antigen gene from Indian isolate of *Leishmania donovani* strain MHOM/IN/DD8,

Another object of this invention is to isolate, characterize and use the immunodominant region of the kinesin related antigen gene from Indian isolate of *Leishmania donovani* strain MHOM/IN/KE16/1998,

Another object of this invention is to identify and do comparative sequence analysis both at nucleic acid and amino acid level of the Indian isolates with that of the *L. chagasi* kinesin immunodominant region,

Still another object is to identify the variations in the 39 amino acid immunodominant repeat region of the kinesin gene isolated from the Indian strains as compared to other reported sequences,

Still another object is to produce polypeptide containing one or more 39 amino acid repeat regionfrom the Indian strains,

Still another object is to produce recombinant polypeptide containing one or more 39 amino acids repeat regionfrom the Indian strains.

Another object of this invention is to provide a method of detecting anti-leishmanial antibody from the leishmania infected patients using the above said polypeptide.

Another object of this invention is to provide a kit containing the polypeptide from the Indian isolates of *Leishmania donovani* to detect anti leishmanial antibody in VL and PKDL patients.

Still another object of this invention is to produce antibody raised against the polypeptides as shown in SEQ ID NO: 5 or SEQ ID NO: 6.

Still another object of this invention is to provide a method for detection of Leishmanial antigens using the above said antibody.

Still another object of this invention is to provide a diagnostic kit consisting of antibody raised against polypeptides as shown in SEQ ID NO: 5 or SEQ ID NO: 6 for detecting Leishmanial antigens .

SUMMARY OF THE INVENTION

Accordingly, the present invention provides polypeptides identified as SEQ ID NO: 5 and SEQ ID NO: 6, isolated from Indian strains of *Leishmania donovani* for the purpose of detecting anti-leishmanial antibodies from infected samples collected from animals or human beings. The invention also provides an ELISA method for detecting the above infection caused by the protozoan parasite of the genus *Leishmania*. The invention further provides a diagnostic kit for detecting leishmaniasis (VL and PKDL) in blood samples. The present invention further provides a diagnostic kit consisting of antibody raised against polypeptides as shown in SEQ ID NO: 5 or SEQ ID NO: 6 for detecting leishmanial antigens

BRIEF DESCRIPTION OF THE DRAWINGS

Figure1:

Cloning of 563bp PCR product in pGEMTE vector (Promega) from the strain MHOM/IN/DD8. Lane 1: 1kb Molecular Weight Marker (MBI Fermentas), Lane 2: Eco R1 digested Plasmid with released 563bp insert, Lane 3: Undigested pGEMTE with Insert, Lane 4: Eco R1 digested Plasmid with released 563bp insert, Lane 5: Undigested pGEMTE with Insert.

Figure 2:

Cloning of 466bp PCR product in pGEMTE vector (Promega) from the strain MHOM/IN/KE16/1998. Lane 1: 1kb Molecular Weight Marker (MBI Fermentas), Lane 2: Eco R1 digested Plasmid with released 466bp insert, Lane 3: Undigested pGEMTE with Insert, Lane 4: Eco R1 digested Plasmid with released 466bp insert, Lane 5: Undigested pGEMTE with Insert.

Figure 3:

The BLAST searches against predicted protein sequence of *L. donovani* strain MHOM/IN/DD8 (SEQ ID NO: 5) using patent division of GenBank database. Figure 3A to 3F, BLAST results against patent database. Query: polypeptide from *L. donovani* strain MHOM/IN/DD8 (SEQ ID NO: 5), subject: patented sequences from US patent number; 5411865, 5719263, 5912166.

Figure 3A - C shows homology between SEQ ID NO: 5 and SEQ ID NO: 1 from US patent 5411865 which is same as the SEQ ID NO: 2 of US patent 5719263.

Figure 3D - F shows homology between SEQ ID NO: 5 and SEQ ID NO: 3 from US patent 5912166.

Figure 4:

Clustal W multiple sequence alignment of the immunodominant repeat regionat the nucleotide level for the sequences, SEQ ID NO: 3 and SEQ ID NO: 4 with that of the immunodominant repeat region of *L. chagasi.* LCIMM: immunodominant repeat region for the species *L. chagasi* (Gen Bank accession No. L07879);

DDIMM (SEQ ID NO: 3) immunodominant repeat region for the species *L. donovani* of Indian strain MHOM/IN/DD8;

KEIMM: (SEQ ID NO: 4) immunodominant repeat region for the Indian strain MHOM/IN/KE16/1998. [---: Represents gaps, identical sequences are shaded black].

Figure 5:

Clustal W multiple sequence alignment of the immunodominant repeat region at the Amino acid level for the sequences, SEQ ID NO: 5 and SEQ ID NO: 6 with that of the immunodominant repeat regionof *L. chagasi.* LCIMM: immunodominant repeat regionfor the species *L.chagasi*, (Gen Bank accession No. L07879). The sequence DDIMM (SEQ ID NO: 5) is the immunodominant repeat region for the species *L.donovani* of Indian strain MHOM/IN/DD8, and KEIMM (SEQ ID NO: 6) is the immunodominant repeat region for the Indian strain MHOM/IN/KE16/1998.

[---: Represents gaps, identical sequences are shaded black and similar sequences are shaded grey].

Figure 6:

Clustal W multiple sequence alignment of the immunodominant repeat region at the Amino acid level for the SEQ ID NO: 5 (DDIMM) with that of the immunodominant repeat region of *L. chagasi.* LCIMM represents the immunodominant repeat region for the species *L. chagasi* (Gen Bank accession No. L07879).

[---: Represents gaps, identical sequences are shaded black and similar sequences are shaded grey].

Figure 7:

Clustal W multiple sequence alignment of the immunodominant repeat region at the Amino acid level for the SEQ ID NO: 5 (DDIMM) and SEQ ID NO: 6 (KEIMM).

[---: Represents gaps, identical sequences are shaded black and similar sequences are shaded grey].

Figure 8:

Immunodominant 39 amino acid repeat unit with intra repeat variation for the strain MHOM/IN/DD8 of Indian isolate of *L. donovani,* as shown in SEQ ID NO: 5. The first 39 amino acid repeat unit is presented and the degeneracies of various amino acid positions between repeats are indicated

Figure 9:

Immunodominant 39 amino acid repeat unit with intra repeat variation for the strain MHOM/IN/KE16/1998 of Indian isolate of *L. donovani*, as shown in SEQ ID NO: 6. The first 39 amino acid repeat unit is presented and the degeneracies of various amino acid positions between repeats are indicated.

Figure 10:

a) The Ni-NTA agarose purified 6X-His tagged recombinant proteins resolved on 12% SDS-PAGE and stained with Coomassie brilliant blue. Lane 1: Pre stained Molecular weight marker (MBI Fermentas, USA), Lane 2: Purified protein from MHOM/IN/DD8 (~29kDA) lane 3: Purified protein from MHOM/IN/KE16/1998(~26kDa).

b) Western blot with Penta anti-His-HRP conjugate for the purified protein. Lane 1: Pre stained Molecular weight marker (MBI Fermentas, USA), Lane 2: Purified protein from MHOM/IN/DD8 (~29kDA) lane 3: Purified protein from MHOM/IN/KE16/1998(~26kDa).

[The purified protein from MHOM/IN/KE16/1998 of predicted molecular weight of ~26kDa has aberrant mobility on SDS-PAGE and was migrating at higher molecular weight ~32kDa.]

Figure 11:

a) The Ni-NTA agarose purified 6X-His tagged recombinant proteins resolved on 12% SDS-PAGE and stained with Coomassie brilliant blue. Lane 1: Pre stained Molecular weight marker (MBI Fermentas, USA), Lane 2: Purified protein from MHOM/IN/DD8 (~29kDA) lane 3: Purified protein from MHOM/IN/KE16/1998(~26kDa).

b) Western blot with KA patient sera for the purified protein. Lane 1: Pre stained Molecular weight marker (MBI Fermentas, USA), Lane 2: Purified protein from MHOM/IN/DD8 (~29kDA) lane 3: Purified protein from MHOM/IN/KE16/1998(~26kDa).

Figure 12:

a) The Ni-NTA agarose purified 6X-His tagged recombinant proteins resolved on 12% SDS-PAGE and stained with Coomassie brilliant blue. Lane 1: Pre stained Molecular weight marker (MBI Fermentas, USA), Lane 2: Purified protein from MHOM/IN/DD8 (~29kDA) lane 3: Purified protein from MHOM/IN/KE16/1998(~26kDa).

b) Western blot with PKDL patient sera for the purified protein. Lane 1: Pre stained Molecular weight marker (MBI Fermentas, USA), Lane 2: Purified protein from MHOM/IN/DD8 (~29kDA) lane 3: Purified protein from MHOM/IN/KE16/1998(~26kDa).

Figure 13:

a) The Ni-NTA agarose purified 6X-His tagged recombinant proteins resolved on 12% SDS-PAGE and stained with Coomassie brilliant blue. Lane 1: Pre stained Molecular weight marker (MBI Fermentas, USA), Lane 2: Purified protein from MHOM/IN/DD8 (~29kDA) lane 3: Purified protein from MHOM/IN/KE16/1998(~26kDa).

b) Western blot with pooled Healthy control patient's sera (N=5) for the purified protein. Lane 1: Pre stained Molecular weight marker (MBI Fermentas, USA), Lane 2: Purified protein from MHOM/IN/DD8 (~29kDA) lane 3: Purified protein from MHOM/IN/KE16/1998(~26kDa).

Figure 14:

a) The Ni-NTA agarose purified 6X-His tagged recombinant proteins resolved on 12% SDS-PAGE and stained with Coomassie brilliant blue. Lane 1: Pre stained Molecular weight marker (MBI Fermentas, USA), Lane 2: Purified protein from MHOM/IN/DD8 (~29kDA) lane 3: Purified protein from MHOM/IN/KE16/1998(~26kDa).

b) Western blot with only secondary antibody control for the purified protein. Lane 1: Pre stained Molecular weight marker (MBI Fermentas, USA), Lane 2: Purified protein from MHOM/IN/DD8 (~29kDA) lane 3: Purified protein from MHOM/IN/KE16/1998(~26kDa).

Figure 15:

The graph shows titer value for different groups of sera on ELISA with purified polypeptide (SEQ ID NO: 5) of MHOM/IN/DD8 origin. The mean OD value with

standard deviation is shown and also depicted in the graph. The different groups included are as follows:

1) Endemic healthy control samples from clinically and serologically (for rK39) negative individuals from an endemic area in Bihar, India,

2). Samples from confirmed VL and PKDL cases (diseased cases) from an endemic area in Bihar, India,

3). Samples from patients positive for HCV,

4) Samples from patients positive for HIV

5). Samples from patients positive for TB,

6) Samples from patients positive for HBs Ag,

7). Non-endemic healthy control samples were obtained from individuals from a non-endemic area (Delhi) for VL.

Figure 16:

The graph shows titer value for different groups of sera on ELISA with purified polypeptide (SEQ ID NO: 6) of MHOM/IN/KE16/1998 origin. The mean OD value with standard deviation is shown and also depicted in the graph. The different groups included are as follows:

1) Endemic healthy control samples from clinically and serologically (for rK39) negative individuals from an endemic area in Bihar, India,

2). Samples from confirmed VL and PKDL cases (diseased cases) from an endemic area in Bihar, India,

3). Samples from patients positive for TB,

4) Samples from patients positive for HCV

5). Samples from patients positive for HIV,

6) Samples from patients positive for HBs Ag,

7). Healthy control samples are from individuals from a non-endemic area (Delhi) for VL.

BRIEF DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID NO: 1 is the nucleotide sequence of full length kinesin related antigen gene from Indian isolate of *L. donovani* strain MHOM/IN/DD8.

SEQ ID NO: 2 is the nucleotide sequence of full length kinesin related antigen gene from Indian isolate of *L. donovani* strain MHOM/IN/KE16/1998.

SEQ ID NO: 3 is the nucleotide sequence encoding immunodominant 39 amino acid repeat polypeptide from Indian isolate of *L. donovani* strain MHOM/IN/DD8

SEQ ID NO: 4 is the nucleotide sequence encoding immunodominant 39 amino acid repeat polypeptide from Indian isolate of *L. donovani* strain MHOM/IN/KE16/1998.

SEQ ID NO: 5 is the amino acid sequence of a 39 amino acid repeat regionantigen from Indian isolate of *L. donovani* strain MHOM/IN/DD8

SEQ ID NO: 6 is the amino acid sequence of a 39 amino acid repeat regionantigen from Indian isolate of *L. donovani* strain MHOM/IN/KE16/1998

SEQ ID NO: 7 is the synthetic oligonucleotide, LKF 93

SEQ ID NO: 8 is the synthetic oligonucleotide, LKR1803

SEQ ID NO: 9 is the synthetic oligonucleotide, LKF 1527

SEQ ID NO: 10 is the synthetic oligonucleotide, LKF 2564

SEQ ID NO: 11 is the synthetic oligonucleotide, LKR 3266

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a recombinant antigen developed from the Indian isolates of *L. donovani*, which is a variant of existing recombinant kinesin related antigen (rK39) for diagnosing VL in India and other geographical areas where *L. donovani* is the causative agent. This recombinant antigen of Indian origin varies upto 60% in the predicted amino acid sequence at the immunodominant repeat epitope to that of the rK39. Recently, the kinesin related antigen gene has been cloned and characterized from *L. chagasi* an, American visceralising species by Burns *et al.;* this led to the development of rK39 antigen, a recombinant protein with 39 amino acid tandem repeats. This above [Burns *et al.,* 1993] antigen from a new world visceralising species *L. chagasi* is reported not sensitive in some highly endemic regions for VL, Sudan and Southern Europe [Zijlstra, E. E., 2001; Jelinek T., 1999] as it is on some other geographical areas, India, Bangladesh, Nepal etc. India carries the majority of the VL population in the global leishmaniasis burden. So, it is very important to have precise and well characterized tools for the diagnosis and other control measurements in India. The applicants have characterized the kinesin related antigen from two strains namely, a) MHOM/IN/DD8 a WHO reference strain originally obtained from a kala-azar patients from Bihar, India and b) MHOM/IN/KE16/1998 obtained from a 10 year old female kala-azar patient from Musafarpur, Bihar, India of *L. donovani* at the sequence level to study, whether the sequence is similar to that one of *L. chagasi* and to rule out the suspicion that, are some cases are going unnoticed in India as in Sudan?

Also it becomes very important to know that, are the various ethnic groups responding differently to the rK39 antigen or it is the sequence divergence at the immunodominant region that causes the difference in sensitivity. In this invention we found that, the sequence of kinesin antigen/polypeptide significantly differs from that of the published *L. chagasi* from which the rK39 was derived.

In the present invention we have also raised antibodies against the kinesin antigen/polypeptide and utilized it for the detection of Leishmanial antigens.

Parasites were initially isolated as Promastigotes in NNN medium from clinical samples of Kala-azar patients and subsequently adapted to grow at 25^0C in Medium 199 containing 10% heat inactivated FCS. For routine maintenance, samples of the inoculum containing parasites were introduced aseptically into culture tubes with 4ml of medium 199 supplemented with 10% FCS. The tubes were placed in cooled incubator at 25^0C and the growth was monitored at regular intervals by microscopy. For mass cultivation of the parasite, samples of inoculum containing parasites were introduced aseptically into 200ml of M199 containing 10%FCS in a 500ml tissue culture flask and incubated in a cooled incubator at 25^0C until mid log phase (7-10 days). The parasites were then harvested and used for nuclear DNA isolation.

The parasites in their mid log phase was harvested by centrifuging at 5000 rpm in a refrigerated centrifuge. Parasite nuclear DNA was isolated following standard protocol with minor modifications [Lu H.G. *et al.*, 1994]. Approximately 1-5 X 10^9 promastigotes were lysed in 10 volumes of lysis buffer (NaCl, 100 mM, Tris-HCl, 10mM (pH 8.0), EDTA 10mM, Proteinase K/ml 100µg, Sarcosyl 1.5%) at 60^0 C for 3 hours. The kinetoplast DNA networks were sedimented by centrifugation at 27,000 X g for 1 hour and resuspended in TE buffer (Tris-HCl (pH 8.0) 10mM, EDTA (pH 8.0) 1mM). The nuclear DNAs were isolated from the supernatants left after sedimentation of the kDNAs. These supernatants were incubated overnight for further digestion of proteins at 65^0 C. The nuclear DNA was subjected to several cycles of phenol/chloroform extractions by adding equal volume of phenol/chloroform mixture, mixing thoroughly followed by sedimentation by centrifugation at 5000 rpm for 15 minutes. The nuclear DNA was precipitated by adding $1/10^{th}$ the volume of 3M-sodium acetate and 2 volumes of 100% ethanol mixed well and incubated at -20^0C for 1 hour. The mixture was sedimented by centrifugation at 5000 rpm for 30 minutes at 4^0C. The pellet was washed with 70% ethanol, dried and resuspended in TE buffer.

The concentration and purity of the DNA was measured by taking OD at 260/280nm.The DNA was stored at -70^{0}C until use.

The PCR for the amplification of kinesin related antigen was performed as below using 50ng of the isolated nuclear DNA and using the following primers in various combinations to amplify the overlapping parts in the kinesin gene. The primes were designed based on the sequence data from the GenBank for the kinesin gene (Accession No. L07879). The available kinesin gene sequence from *L. chagasi* is 3319 bp in length and has an ORF from position 454 with a putative ATG starting codon and extends until the last base at position 3319. This gene has 5' non repeat region from the starting codon at position 2563 and a conserved immunodominant repeat domain from base 2564 to till the end in the 3'. The repetitive epitope of 39 amino acid is part of the kinesin gene at the 3'end from the base position 2564 to 3319. This repetitive epitope has 117bp tandem repeat at 6.5 copies reported and extending upto 3 to 4kb in the 3' end. The highly conserved short stretches of sequences at various positions were considered for primer designing. The whole 3.33kb gene of the *L. chagasi* was considered and we attempted to amplify the open reading frame (ORF) from the position 454 to 3319bp. The main focus being on the immunodominant tandem repeats of 117bp size; however we attempted to amplify and characterize complete ORF with as many repeats as possible. Three forward and two reverse primers were designed to amplify this gene detailed as follows; the primer LKF93 (SEQ ID NO: 7) is upstream to the predicted starting codon at 455bp and it covered 362bp 5' to the starting of ORF. The primer LKF1527 (SEQ ID NO:9) is another forward primer, which starts from the position at the base 1527 from the base one i.e. it covers 1073 bases from the beginning of the ORF. The third forward primer is LKF 2564 (SEQ ID NO: 10) that is intended to amplify the immunodominant tandem repeats in combination with the reverse primer LKR3266 (SEQ ID NO: 11). The first reverse primer is LKR 1803 (SEQ ID NO: 8), which includes an overlapping region of 276bp with that of the second primer set with another reverse primer LKR3266 (SEQ ID NO: 11). The primer sequences are given in the SEQ ID NO: 7 to SEQ ID NO: 11. The first PCR primer set LKF 93 (SEQ ID NO:7) and LKR 1803 (SEQ ID NO:8) amplified a 1.71 kb fragment from the position at 93 base at the 5' end of the kinesin gene to the position 1803bp in the 3' position. The second PCR primer set LKF 1527 (SEQ ID NO: 9) and LKR 3266 (SEQ ID NO: 11) amplified a 1.15 kb fragment with one 117 bp tandem repeat. This PCR amplicons had 276bp overlapping nucleotides at

the 5' end with that of first PCR product amplified. The third PCR primer set LKF 2564 (SEQ ID NO: 10) and LKR 3266 (SEQ ID NO: 11) amplified the immunodominant 117 tandem repeat region. This third primer set amplified a fragment of size approximately 470 bp corresponding the four 117bp tandem repeat for MHOM/IN/KE16/1998 and approximately 590bp size product corresponding five immunodominant tandem repeat for the strain MHOM/IN/DD8. All the PCR products were purified as described below and cloned in pGEMTE cloning vector and sequenced in an automated DNA sequencer.

By using aerosol free pipette tips and keeping the pre- and post – PCR products separately, amplicons carry over contamination was avoided. All PCR reactions were performed using standard protocols (Sambrook *et al.,* 1989) with a set of negative controls.

10X *Taq* buffer	5.00µl
10mM dNTPs	1.00µl
Primer Forward (25µM)	1.00µl
Primer Reverse (25µM)	1.00µl
Taq DNA polymerase (5U/µl) (Promega, USA)	0.25µl
Template DNA	2.00µl
Sterile water to make up the volume to	50.00µl

The tubes were kept in thermal cycler (MJ Research, USA) and incubated at 95^0 C for 5 minutes followed by 35 cycles of amplification.

The amplified PCR products were resolved on agarose gel electrophoresis. The gel was visualized under ultraviolet transilluminator (UVP) and photographed using a Polaroid camera.

The kinesin gene following amplification by PCR was cloned in a TA cloning vector as below. The PCR amplified DNA were resolved on agarose gel and the portion containing the band of interest was excised with a sterile scalpel. The DNA was eluted from the gel using gel elution kit (Qiagen, Germany) following the manufacturer's protocol. Concentration of eluted DNA was measured by absorbance at 260nm in spectrophotometer.

The gel purified PCR product of interest was ligated directly in pGEMT-Easy vector. In a 1.5ml micro centrifuge tube the following components were added.

pGEM-T Easy	1.00µl
10X ligation buffer	1.00µl
DNA (200ng)	5.00µl
T4 DNA Ligase (2U/µl)	1.00µl
Water to make the volume upto	10.00µl

After mixing gently, the tubes were incubated at 4^0 C overnight and heated for 10minutes at 70^0 C. The samples were stored at -20^0 C until transformation.

The ligated mixture was then transformed by heat sock treatment. The competent cells were prepared by using calcium chloride method [Sambrook *et al.*, 1989]. A single colony of *E. coli* was inoculated in 5ml of LB medium and incubated at 37^0C overnight with shaking (200rpm). Next day fresh stock of 100ml LB medium was inoculated with 1ml of the overnight culture and incubated at 37^0C with continuous shaking until the O.D. reached 0.6 at 600nm. The culture was chilled on ice for 30minutes and the cells were harvested by centrifugation at 4000g, 4^0C for 10 min. The cell pellet was resuspended in $1/10^{th}$ volume of ice cold filtered sterile 50mM $CaCl_2$ and kept on ice for 30 min. The cells were pelleted down and finally resuspended in $1/25^{th}$ volume of ice-cold 50mM $CaCl_2$ (with 20% V/V of autoclaved glycerol) and either stored at -70^0C in aliquots of 200µl or used immediately for transformation.

Approximately 5µl of ligation mixture was gently mixed with competent cell (200µl) and incubated in ice for 30 min. After incubation the cells were placed in water bath set at 42^0C for 90 seconds (heat shock) and immediately transferred to ice. 800µl of LB medium was added to the cells and kept at 37^0C for 90 minutes with shaking (150rpm). The cells were plated with 16µl of X-gal and 10µl of 1M IPTG on LB agar plates containing 50µg/ml of ampicillin. The plates were incubated at 37^0C for 12-16 hours. The white colonies were selected and checked for the insert.

For screening, the Plasmid was isolated by rapid boiling method [Holmes and Quigley, 1981]. The colonies were picked up with sterile toothpick and inoculated in 5ml of fresh medium. The cultures grown overnight were pelleted and the cells were resuspended in 500µl of STET buffer (Sucrose 8% (w/v), Tris-HCl, (pH 8.0), 50mM, EDTA Na_2, (pH 8.0) 50mM, Triton-X 100 5% (w/v) followed by lysis with 40µl of lysozyme. The mixture was vortexed well and incubated in a boiling water bath for 90 seconds. The bacterial debris and chromosomal DNA were removed by centrifugation

at 12000g for 10 minutes. The supernatant was mixed with 400µl of isopropanol and 200µl of 7.5 M ammonium acetate. The Plasmid DNA was pelleted by centrifugation. The dried pellet was resuspended in 100µl of sterile water, mixed with 50µl of 7.5 M ammonium acetate and incubated on ice for 30 minutes. The samples were centrifuged at 4^0C for 10 minutes and Plasmid DNA was precipitated with ethanol. The pure DNA pellet obtained after centrifugation was washed twice with 70% ethanol dried and dissolved in 50µl of sterile water.

The restriction analysis of the recombinant Plasmid was done using appropriate restriction enzyme sites flanking the multiple cloning site of the vectors. The reaction was set as follows:

Plasmid DNA	8.00µl (2µg)
10X reaction buffer	2.00µl
RNase A (10mg/ml)	5.00µl
Restriction Enzyme	5 Units
Sterile water to make the volume up to	20.00µl

The reaction was incubated at 37^0C for 6-8 hours and the products were analyzed on 1.5% agarose gel along with standard molecular weight markers. The positive clones containing the insert as detected by the restriction digestion were used for sequencing and preserved as glycerol stock.

All the sequencing was done by chain termination method [Sanger *et al.*, 1977] in an automated DNA sequencer, ABI Prism version 7.0. The Sequences were analysed using various software like DNASIS, Lasergene: edit; Megalign etc (DNA star Inc.), Clustal W (multiple alignment of various sequence files). All the sequences obtained were aligned to form continuous stretch by using Seqman II (Lasergene package). Along with this, the sequence was searched for homology by using BLAST [Altschul *et al.*, 1997] option from various websites.

The full-length kinesin sequence obtained after assembling the sequence was presented in the SEQ ID NO: 1 for the strain MHOM/IN/DD8, it was found to be 3016 bp in length with an open reading frame of 2670bp. The length of the sequence for the strain MHOM/IN/KE16/1998 was found to be 2937bp with a long open reading frame of 2577bp (SEQ ID NO: 2). The sequence analysis revealed that the size of the PCR product amplifying the immunodominant region was 563bp for the strain MHOM/IN/DD8 (figure1) corresponding to 4.8 tandem repeats of 117bp corresponding to 4.8 units of 39 amino acid repeats and 466 bp for the strain

MHOM/IN/KE16/1998 (figure 2) corresponding to 4 tandem repeats of 117bp (4 units of 39 amino acid repeats).

Further, the sequence analysis shows significant variation in both at DNA level as well as in the predicted amino acid sequence from that of the *L. chagasi* sequence. The strain MHOM/IN/DD8 shows enormous variation from that of *L. chagasi* as well as from the strain MHOM/IN/KE16/1998. The blast search for predicted protein sequence of the strain MHOM/IN/DD8 (SEQ ID NO: 5) using non-redundant and patent division of GenBank database reveals only up to 38% identity with that of published *L. chagasi* and US patent nos. i) US 5411865 and ii) US 5912166 respectively figure 3.

The clustal W multiple sequence alignment of the immunodominant repeat regionat the nucleotide level for the *L. donovani* strains MHOM/IN/DD8 and MHOM/IN/KE16/1998 (SEQ ID NO: 3 and SEQ ID NO: 4) with that of the immunodominant repeat regionof *L. chagasi* was done and presented in the figure 4. The identities are shadowed dark. The multiple sequence alignment of the immunodominant repeat regionat the amino acid level between *L. donovani* (SEQ ID NO: 5 and SEQ ID NO: 6) *L. chagasi* is presented in figure 5. Multiple sequence alignment of the immunodominant repeat regionat the amino acid level for the *L. donovani* strain MHOM/IN/DD8 (SEQ ID NO: 5) with that of the immunodominant repeat regionof *L. chagasi* is presented in the figure 6. Sequence alignments of the immunodominant repeat regionat the amino acid level for the two *L. donovani* strains MHOM/IN/DD8 and MHOM/IN/KE16/1998 (SEQ ID NO: 5 and SEQ ID NO: 6) is presented in figure 7. Immunodominant 39 amino acid repeats unit with intra repeat variation for the Indian isolates of *L. donovani,* strains MHOM/IN/DD8 and MHOM/IN/KE16/1998 (SEQ ID NO: 5 and SEQ ID NO: 6) are presented in figure 8 and figure 9. The sequence analysis reveals that, the amino acids varies enormously having many substitution both conserved and variable at many positions in the 39 amino acid sequence reported for *L. chagasi.* At least five amino acids for *L. donovani* are different to *L. chagasi* at positions 18, 27, 29, 32 and 38 indicated by "*" in the table 1 below,

After analyzing the sequences insilico we found a significant variation in the amino acid sequence at the immunodominant region of the strain MHOM/IN/DD8 with that of the *L. chagasi.* Then, to confirm whether the sequence variation causes any effect on the performance of the antigen to detect anti leishmanial antibodies, we

expressed the immunodominant region from the two Indian *Leishmania donovani* isolates in *E. coli* expression vector pRSET (Invitrogen) as 6X-His tagged protein. We have expressed various combinations of the immunodominant repeat like one, two, three, four, 4.8 units and one repeat with the 889 nucleotides upstream to the immunodominant domain. We found that recombinant peptides with four and 4.8 repeat units having higher sensitivity and specificity. Thus, our work was focused on polypeptides containing 4.0 and 4.8 repeat units of 39 amino acids.

The *E. coli* expression vector pRSET C (Invitrogen, Netherlands) was completely digested with PstI and NcoI restriction enzymes at 37^0 C overnight. The digested plasmid was fractionated on agarose gel to remove the stuffer fragment. The plasmid band was excised and eluted by Qiagen gel elution system and stored in -20^0C until further use.

The insert was released from the recombinant pGEM-TE plasmid carrying the immunodominant tandem repeats (pGEM-TEasy/DD8/LKF2564 and pGEM-TEasy/KE16/LKF2564) by digesting it with PstI and NcoI. The inserts was excised from the gel, eluted by Qiagen gel elution system and ligated in PstI and NcoI digested pRSET C vector in-frame and transformed to BL21 competent cells by the standard protocol [Sambrook *et al.,* 1989]. The clones were selected on the ampicillin plates and plasmid DNA was isolated and digested with PstI and NcoI to check for the insert. The clones, which released the insert, were selected for protein induction.

Table 1

Amino Acid Position	MHOM/IN/DD8 (SEQ ID NO: 5)	MHOM/IN/KE16/1998 (SEQ ID NO: 6)	*L. chagasi* (Reed USPTO: 5,411,865)
1	K, L, A & S	L	L
2	E	E	E
3	Q, G, V, A	Q	Q
4	Q, R	Q, R	Q
5	L, A	L	L
6	R, A	R	R
7	D, E	D, E	D, E
8	S, L	S	S
9	E, A	E	E
10	T, R, G & A	E, A	E, A
11	R, K, V & Q	R, H	R
12	A, L	A	A
13	A, E, K	A	A
14	E, A & S	E	E
15	L, T, A	L	L
16	K, A	M, K & A	A
17	A, S	R, A & S	S
18*	**E, A & V**	**K, Q**	**Q**
19	L, K	L	L
20	E, N, T & S	E	E
21	A, L, & S	A, S	A, S
22	T, V & M	T	T
23	A, E	A	A, T
24	A, Q	A	A
25	A, D & E	A	A
26	K, R	K	K
27*	**T, E**	**S, T**	**M, S**
28	S, R & N	S	S
29*	**V, T**	**A**	**A**
30	E, R	E	R
31	Q, A	Q	Q
32*	**E, T & A**	**D**	**D**
33	R, L	R	R
34	E	E	E
35	K, E & Q	N	N, Q
36	T, R & Q	T	T
37	R, L	R	R
38*	**T, R**	**A**	**A**
39	A, I, E & L	T, A	T, A

Then, a single recombinant *E. coli* (BL 21) colony containing the insert was inoculated in 2ml of SOB containing Ampicillin (50μg/ml) and incubated overnight at 37^0C with shaking (225rpm). The next day, 25ml of SOB was inoculated with the overnight culture and allowed to grow at 37^0C with shaking to an OD_{600} of 0.6. Removed 1ml aliquot of cells, centrifuged and frozen in -20^0C. To the culture added IPTG to a final concentration of 1mM and incubated at 37^0C with shaking. A time course of expression to determine the optimal induction time for maximum expression of protein was done by taking aliquot of cells at 1, 2, 3, 4 & 5 hours after induction with IPTG and analyzed by SDS-PAGE and western blot. Further, to determine the protein solubility, a single recombinant *E. coli* (BL 21) colony containing the insert was inoculated in 2ml of SOB containing Ampicillin (50μg/ml) and incubated overnight at 37^0C with shaking (225rpm). The next day, 25ml of SOB was inoculated with the overnight culture and allowed to grow at 37^0C with shaking to an OD_{600} of 0.6. Removed 1ml aliquot of cells, centrifuged and frozen in -20^0C. To the culture added IPTG to a final concentration of 1mM and incubated at 37^0C with shaking for additional 4 hours. The cells were harvested by centrifugation at 4000xg for 20 minutes and the pellet was resuspended lysis buffer for purification under native conditions (Qiagen, Germny) and lysed by sonication, 6 x 10 s with 10 s pauses at 200–300 W. The lysate was centrifuged in a refrigerated centrifuge for 10 minutes at 10,000xg. The supernatant was transferred to a fresh tube and the pellet was resuspended in lysis buffer and preserved on ice. The pellet and the supernatant was mixed with 2 X SDS-PAGE sample buffers separately and analyzed by using 12 % SDS-PAGE and western blot. The results show that the protein is detectable in the soluble fraction and thus can be purified under native conditions.

The maximum protein-producing clones were inoculated in 20ml of SOB containing 100μg/ml Ampicillin and grew overnight at 37^0C with vigorous shaking. The next day, 1 liter of SOB was inoculated with 1:50 noninduced overnight culture and allowed to grow at 37^0C with shaking until an OD_{600} of 0.6 is reached. A 5 ml aliquot of culture was taken immediately before induction. The cells were induced by adding IPTG to a final concentration of 1mM and continued incubation at 37^0C for 4 hours. The cells were harvested by centrifugation at 4000xg for 20 minutes and stored in -20^0C until purification.

The recombinant protein was purified using Ni-NTA agarose column (Qiagen, Germany) under native conditions following the manufacturer's protocol. The cell

pellets were thawed for 15 minutes on ice and resuspended in lysis buffer at 5ml per gram wet weight. To this lysozyme was added at 1mg/ml and incubated for 30 minutes on ice and sonicated on ice using six 10 s bursts at 200–300 W with a 10 s cooling period between each burst. The lysate was centrifuged at 10,000x g for 30 minutes at 4^0C to sediment the cellular debris. The supernatant was saved. An aliquot of 50µl supernatant was taken and stored in -20^0C for analysis later. Added 1ml of the 50% Ni-NTA slurry to 4 ml of cleared lysate and mixed gently by shaking (200rpm on a rotary shaker) at 4^0C for 60 minutes. The lysate-Ni-NTA mixture was loaded on to a column with the bottom outlet capped. The flow -throw was collected after removing the bottom cap and the column was washed twice with 4 ml of wash buffer and the fractions were collected for SDS-PAGE analysis. The protein was eluted 4 times with 0.5ml of elution buffer and the eluate was collected in 4 tubes and analyzed by SDS-PAGE. The protein expression after purification was found to be 4mg/liter.

For analysis of the expressed protein, Mini gel electrophoresis unit (Bangalore Genei, India) was used. The SDS-PAGE was carried out as per defined protocols [Laemmeli, 1970]. A 10% resolving gel was prepared by mixing the following components: 2.66ml water, 1.562ml 30% acrylamide, 1.250ml 1.5M Tris-HCl, 50µl 10% APS, 10% TEMED. The gel top was with ~ 400µl of water saturated butanol and allowed to polymerize. After polymerization, butanol was drained off and washed with distilled water. Then, 2ml of stacking gel mix (1.25ml water, 250µl 30% acrylamide, 500µl 0.5M Tris-HCl, 30µl 10% APS and 5µl TEMED) was poured carefully avoiding any air bubble on the top of the resolving gel. The comb was inserted in the stacking gel portion and left for polymerization. Following the polymerization the wells were washed with excess amount of water. The samples were applied to the defined wells in the stacking gel and electrophoresis carried out at a constant voltage (60V for stacking and 100V for resolving gel). Standard molecular protein markers (MBI) and control (vector protein) were run alongside the samples. The completed gel was then stained in Coomassie brilliant blue stain, destained and visualized against white light.

The purified protein from the strain MHOM/IN/DD8 migrated at a size of 29kDa in correlation to the predicted molecular weight and the predicted molecular weight for the strain MHOM/IN/KE16/1998 was 26kDa but the purified protein from this strain had aberrant PAGE mobility and was migrating at higher molecular weight ~32kDa (figure 10a). The reported antigens are entirely different from the *Leishmania chagasi* 230kDa antigen. The antigen varies significantly, more than 60% in its

predicted amino acid sequence to that of K39 repeat antigen from *L. chagasi.* Also disclosed are DNA encoding the 29kD and 26kD antigen and therapeutic and vaccine compositions comprising the antigens.

Purified recombinant polypeptides from the strains MHOM/IN/DD8 and MHOM/IN/KE16/1998 was used for immunization in rabbits. 100 micro gram of each polypeptide was emulsified with equal volume of Freund's complete adjuvant (Sigma, USA). The mixture was injected intradermally at multiple sites. Three booster doses of the same amount of antigen emulsified with incomplete Freund's adjuvant were given at 30-day intervals. Prior to the first immunization, preimmune serum was collected and tested on western blots. Blood was collected aseptically from the immunized animals 12 days after the last booster, sera was separated and stored on -70^0 C until use.

The *E. coli* expressed protein was resolved on to 12% SDS-PAGE followed by electro transfer to nitrocellulose membrane [Towbin *et al.,* 1976]. The transfer was carried out using transfer buffer (Tris base 3.08g, Glycine 14.66g, Methanol 200ml, Water to the final volume of 1000ml) in Bio-Rad semi dry transfer unit. For transfer, 15V was applied and allowed to run for 45 minutes at room temperature. After transfer the membrane was put in a blocking solution containing 2.5% BSA in phosphate buffered saline (PBS, NaCl 8.00g, KCl 0.20g, NaH_2PO_4 1.44g, KH_2PO_4 0.24g, pH 7.4). The blocked membrane was washed three times with PBS containing 0.02% Tween-20 (PBS-T). The membrane was incubated with kala-azar patient serum (1:200 diluted in PBS) for 1 hour at 25^0C. Further it was washed 4 times in PBS-T and incubated in 1:4000 diluted anti-human IgG antibodies, conjugated with alkaline phosphatase for 45 minutes at room temperature. After several washes with PBS, the membrane was developed by addition of BCIP-NBT (Amresco, USA).

The purified antigens were first run on SDS-PAGE and the subjected to immunoblot with penta-anti-his HRP conjugate antibody (figure 10a and 10b). To study the antigenicity of the purified proteins, SDS-PAGE followed by immunoblot was carried out with kala-azar patient's sera (figure 11a and 11b) and immunoblot with PKDL patient's sera (figure 12a and 12b) followed by western with pooled (n=5) healthy individuals sera as control was done and presented in figure 13a and 13b. Also, immunoblot with only AP-labelled anti-human secondary antibody was done as shown in figure 14a and 14b. The results of the immunoblot and sequence analysis

reveal that, the recombinant antigens from *L. donovani* are specific in spite of having different epitope.

After protein estimation by BCA method (Sigma, the purified antigen was taken for evaluation by ELISA. The ELISA was standardized initially at different parameters with appropriate serum and reagent controls. The ELISA at 50ng/well and 1:100 dilutions of sera was chosen optimal and used the same conditions for ELISA with two antigens from *L. donovani* viz. MHOM/IN/DD8 and MHOM/IN/ KE16/1998. In each plate one positive control (parasitologically and serologically positive for rK39) and one negative control with sera from non-endemic region another with reagent control was included. The plates were coated as below; Polystyrene micro titer plates with 96 flat-bottom wells were coated with antigen following standard protocol with minor modifications [Singh S *et al.*, 1995] by adding 50ng of purified kinesin antigen in 200µl of 0.1M bicarbonate buffer, pH 9.2. The plates were covered and incubated overnight at 4^0C. The antigen solution was then removed and plates were washed 3 times in PBST (PBS with 0.05% Tween 20). The wells were then blocked with 200µl of 1% BSA for 1 hour, washed 3 times with PBST. The plates were dried at room temperature, sealed, and stored at 4^0C until use. The sensitized plates were incubated for 2 hours with 50µl of patient serum diluted from 1:100 to the end point in PBST. The wells were washed again with PBST and incubated with 50µl of goat anti-human IgG conjugated with alkaline phosphatase at 10^{-3} dilution for another 2 hour, followed by washing 3 times with PBST. After incubation for 30 minutes at 37^0C with 50µl of p-nitrophenylphosphate in diethylamine buffer, the reaction was stopped with 50µl of 3N NaOH. The optical density of each well was measured at 450nm in a Tritrius plate reader. The antibody titers to the recombinant protein from MHOM/IN/DD8 (SEQ ID NO: 5) were considerably lower to that of MHOM/IN/KE16/1998 (SEQ ID NO: 6) for the same group of samples. The preliminary studies with 72 Endemic and 80 non-endemic healthy controls, 92 Tuberculosis positive, 32 HIV positive, 31 HCV positive and 27 HbsAg positive sera showed no cross reactivity. However, the rK39 positive previously confirmed cases of 10 VL and 10 PKDL sera show high antibody titer with both the antigens at 50ng/ well concentration. The data show that, these two well-characterized antigens from the Indian strains will be highly useful for the Kala-azar diagnosis in India.

The figure 15 depicts the mean titer value of different samples subjected to ELISA using purified antigen of MHOM/IN/DD8 origin and the figure 16 show the mean titer value of different set of samples for the purified antigen of MHOM/IN/KE16/1998 origin.

Leishmania Antigen Detection using antibody

Purified recombinant polypeptides from the strains SEQ ID NO: 5 and SEQ ID NO: 6 were used for immunization in rabbits. 100 micro gram of each polypeptide was emulsified with equal volume of Freund's complete adjuvant (Sigma, USA). The mixture was injected intradermally at multiple sites. Three booster doses of the same amount of antigen emulsified with incomplete Freund's adjuvant were given at 30-day intervals. Prior to the first immunization, preimmune serum was collected and tested on western blots. Blood was collected aseptically from the immunized animals 12 days after the last booster, sera were separated and the antibody present in the sera was affinity purified and stored at 4^0C until use.

Polystyrene micro titer plates with 96 wells were coated by diluting the antibody stored at 4^0C in coating buffer (pH 9.2) [1.59 g sodium carbonate (Na_2CO_3), 2.93 g sodium bicarbonate ($NaHCO_3$), 0.20 g sodium azide (NaN_3), dissolved in 900 ml H_2O, adjusted pH to 9.6 with HCl and make up to 1 L] and added 200 µl to each well of a microtitre plate. The plates were incubated at 37 °C for 4 h and washed with PBS-Tween, three times. The plates dried by tapping upside down on tissue paper. The antibody coated to the solid support then was used for the detection of Leishmania antigen this method called a double antibody or sandwich ELISA, and was performed as below. To each well added 200 µl aliquots of the test sample and incubated for 4-hours at 37 °C the plates are washed three times with PBST and then incubated with antibody conjugate and incubated at 37 °C for 2 hours washed three times with PBST and added 200 µl aliquots of substrate to each well and incubated at room temperature for 60min. The optical density of each well was measured at 405nm in a Tritrius® plate reader. The sandwich ELISA measures the amount of antigen between two layers of antibodies. The leishmania antigen to be measured contains at least two antigenic sites, capable of binding to antibody, since at least two antibodies act in the sandwich. So sandwich assays are restricted to the quantitation of multivalent antigens such as proteins or polysaccharides. Sandwich ELISA for quantitation of antigens are especially valuable when the concentration of antigens is low and/or they are contained in high concentrations of contaminating protein

The following examples illustrate the invention, which should not be construed to limit the scope of the invention.

EXAMPLE 1:

This example illustrates the cloning of kinesin immunodominant repeat region from Indian isolates of *L. donovani*. A PCR cloning strategy was followed using a primer set LKF 2564 and LKR 3266 (SEQ ID NO: 10 and SEQ ID NO: 11 respectively) to amplify the immunodominant 117 tandem repeat region. This primer set amplified a fragment of size approximately 470 bp corresponding to the four 117bp tandem repeat from *L. donovani* strain MHOM/IN/KE16/1998 and approximately 590bp size product corresponding five immunodominant tandem repeat from *L. donovani* strain MHOM/IN/DD8. The kinesin gene following amplification by PCR was cloned in a TA cloning vector as below. The PCR amplified DNA were resolved on agarose gel and the portion containing the band of interest was excised with a sterile scalpel. The DNA was eluted from the gel using gel elution kit (Qiagen, Germany) following the manufacturer's protocol. Concentration of eluted DNA was measured by absorbance at 260nm in spectrophotometer.

The gel purified PCR product of interest was ligated directly in pGEMT-Easy vector (Promega, USA). In a 1.5ml micro centrifuge tube the following components were added.

pGEM-T Easy (100ng/μl)	1.00μl
10X ligation buffer	1.00μl
DNA (200ng)	5.00μl
T4 DNA Ligase (2U/μl)	1.00μl
Water to make the volume upto	10.00μl

After mixing gently, the tubes were incubated at 4^0 C overnight and heated for 10minutes at 70^0 C. The samples were stored at -20^0 C until transformation.

The ligated mixture was then transformed by heat sock treatment. The competent cells were prepared by using calcium chloride method [Sambrook *et al.*, 1989]. A single colony of *E. coli* was inoculated in 5ml of LB medium and incubated at 37^0C overnight with shaking (200rpm). Next day fresh stock of 100ml LB medium was inoculated with 1ml of the overnight culture and incubated at 37^0C with continuous shaking until the O.D. reached 0.6 at 600nm. The culture was chilled on ice for

30minutes and the cells were harvested by centrifugation at 4000g, 4^0C for 10 min. The cell pellet was resuspended in $1/10^{th}$ volume of ice cold filtered sterile 50mM $CaCl_2$ and kept on ice for 30 min. The cells were pelleted down and finally resuspended in $1/25^{th}$ volume of ice-cold 50mM $CaCl_2$ (with 20% V/V of autoclaved glycerol) and either stored at -70^0C in aliquots of 200µl or used immediately for transformation.

Approximately 5µl of ligation mixture was gently mixed with competent cell (200µl) and incubated in ice for 30 min. After incubation the cells were placed in water bath set at 42^0C for 90 seconds (heat shock) and immediately transferred to ice. 800µl of LB medium was added to the cells and kept at 37^0C for 90 minutes with shaking (150rpm). The cells were plated with 16µl of X-gal and 10µl of 1M IPTG on LB agar plates containing 50µg/ml of ampicillin. The plates were incubated at 37^0C for 12-16 hours. The white colonies were selected and checked for the insert. The recombinant clones were further characterized by restriction mapping and sequencing. All the sequencing was done by chain termination method [Sanger *et al.*, 1977] in an automated DNA sequencer, ABI Prism version 7.0. The Sequences were analysed using various software like DNASIS, Lasergene: edit; Megalign etc (DNA star Inc.), Clustal W (multiple alignment of various sequence files). All the sequences obtained were aligned to form continuous stretch by using Seqman II (Lasergene package).

Along with this, the sequence was searched for homology by using BLAST [Altschul *et al.*, 1997] option from various websites.

The full-length kinesin sequence obtained after assembling the sequence was presented in the SEQ ID NO: 1 for the strain MHOM/IN/DD8, it was found to be 3016 bp in length with an open reading frame of 2670bp. The length of the sequence for the strain MHOM/IN/KE16/1998 was found to be 2937bp with a long open reading frame of 2577bp (SEQ ID NO: 2). The sequence analysis revealed that the size of the PCR product amplifying the immunodominant region was 563bp for the strain MHOM/IN/DD8 (figure 1) corresponding to 4.8 tandem repeats of 117bp and 466 bp for the strain MHOM/IN/KE16/1998 (figure 2) corresponding to 4 tandem repeats of 117bp.

Further, the sequence analysis shows significant variation in both at DNA level as well as in the predicted amino acid sequence from that of the reported *L. chagasi* sequence (GenBank, Accession No. L07879). The strain MHOM/IN/DD8 shows significant variation from that of *L. chagasi* as well as from the strain

MHOM/IN/KE16/1998. The blast search for predicted protein sequence of the strain MHOM/IN/DD8 (SEQ ID NO: 5) using non-redundant and patent division of GenBank database reveals only up to 38% identity with that of published *L. chagasi* and US patent nos. i) US 5411865 and ii) US 5912166 respectively figure 3.

The clustal W multiple sequence alignment of the immunodominant repeat regionat the nucleotide level for the *L. donovani* strains MHOM/IN/DD8 and MHOM/IN/KE16/1998 (SEQ ID NO: 3 and SEQ ID NO: 4) with that of the immunodominant repeat regionof *L. chagasi* (GenBank, Accession No. L07879) was done and presented in the figure 4. The identities are shadowed dark. The multiple sequence alignment of the immunodominant repeat regionat the amino acid level between *L. donovani* (SEQ ID NO: 5 and SEQ ID NO: 6) and *L. chagasi* is presented in figure 5. Multiple sequence alignment of the immunodominant repeat regionat the amino acid level for the *L. donovani* strain MHOM/IN/DD8 (SEQ ID NO: 5) with that of the immunodominant repeat regionof *L. chagasi* is presented in the figure 6. Sequence alignments of the immunodominant repeat regionat the amino acid level for the two *L. donovani* strains MHOM/IN/DD8 and MHOM/IN/KE16/1998 (SEQ ID NO: 5 and SEQ ID NO: 6) is presented in figure 7. Immunodominant 39 amino acid repeats unit with intra repeat variation for the Indian isolates of *L. donovani,* strains MHOM/IN/DD8 and MHOM/IN/KE16/1998 (SEQ ID NO: 5 and SEQ ID: 6) are presented in figure 8 and figure 9. The sequence analysis reveals that, the amino acids vary significantly having many substitutions. These substitutions are conserved or are variable at many positions in the 39 amino acid repeated region reported for *L. chagasi*. At least five amino acids for *L. donovani* are different to *L. chagasi* at positions 18, 27, 29, 32 and 38 indicated by "*" in the table 1.

EXAMPLE 2:

This example illustrates the reactivity of patient sera to recognize the recombinant *L. donovani* antigens. The insert was released from the recombinant pGEM-TE plasmid carrying the immunodominant tandem repeats (pGEM-TEasy/DD8/LKF2564 and pGEM-TEasy/KE16/LKF2564) by digesting it with PstI and NcoI. The inserts was excised from the gel, eluted by Qiagen gel elution system and ligated in PstI and NcoI digested pRSET C vector in-frame and transformed to BL21 competent cells by the standard protocol [Sambrook *et al.,* 1989]. The clones were selected on the ampicillin plates and plasmid DNA was isolated and digested

with PstI and NcoI to check for the insert. The clones, which released the insert, were selected for protein expression. The maximum protein-producing clones were inoculated in 20ml of SOB medium containing 100μg/ml Ampicillin and grown overnight at 37^{0}C with vigorous shaking. The next day, 1 liter of SOB medium was inoculated with 1:50 noninduced overnight culture and allowed to grow at 37^{0}C with shaking until an OD_{600} of 0.6 is reached. A 5 ml aliquot of culture was taken immediately before induction. The cells were induced by adding IPTG to a final concentration of 1mM and continued incubation at 37^{0}C for 4 hours. The cells were harvested by centrifugation at 4000xg for 20 minutes and stored in –20^{0}C until purification. The recombinant polypeptide was purified using Ni-NTA agarose column (Qiagen, Germany) under native conditions following the manufacturer's protocol. The purified protein from the strain MHOM/IN/DD8 migrated at a size of 29kDa (figure 10a, lane 2) that correlates with the predicted molecular weight. The purified antigens were first run on SDS-PAGE (figure 10a, 11a, 12a and 13a) and the subjected to immunoblot with penta-anti-his HRP conjugate antibody (figure 10b), kala-azar patient's sera (figure 11b), PKDL patient's sera (figure 12b) followed by western with pooled (n=5) healthy individuals sera (figure 13b), with only AP-labelled anti-human secondary antibody (figure 14b) were done. The results of the immunoblot and sequence analysis reveal that, the recombinant antigens from *L. donovani* are specific in spite of having different epitope.

EXAMPLE 3:

This example illustrates the reactivity of patient sera to recognize the *L. donovani* antigens. Protein estimation was done by BCA method (Sigma, USA) and the purified antigens were used for ELISA. The ELISA was standardized initially at different parameters with appropriate serum and reagent controls. The ELISA at 50ng/well and 1:100 dilutions of sera was chosen optimal and used the same conditions for ELISA with two recombinant polypeptides from *L. donovani* viz. MHOM/IN/DD8 (SEQ ID NO: 5) and MHOM/IN/KE16/1998 (SEQ ID NO: 6). In each plate one positive control (parasitologically and serologically positive for rK39) and one negative control with sera from non-endemic region and another with reagent control was included. The plates were coated as below; Polystyrene micro titer plates with 96 flat-bottom wells were coated with antigen following the protocol as reported by Singh S *et al.,* [2002] with minor modifications by adding 50ng of purified kinesin antigen in 200μl of 0.1M bicarbonate buffer, pH 9.2. The plates were covered and

incubated overnight at 4^0C. The antigen solution was then removed and plates were washed 3 times in PBST (PBS with 0.05% Tween 20). The wells were then blocked with 200µl of 1% BSA for 1 hour, washed 3 times with PBST. The plates were dried at room temperature, sealed, and stored at 4^0C until use. The sensitized plates were incubated for 2 hours with 50µl of patient serum diluted from 1:100 to the end point in PBST. The wells were washed thrice with PBST and incubated with 50µl of goat anti-human IgG conjugated with alkaline phosphatase at 10^{-3} dilution for another 2 hour, followed by washing 3 times with PBST. After incubation for 30 minutes at 37^0C with 50µl of p-nitrophenylphosphate in diethylamine buffer, the reaction was stopped with 50µl of 3N NaOH. The optical density of each well was measured at 450nm in a Tritrius® plate reader. The antibody titers to the recombinant protein from MHOM/IN/DD8 (SEQ ID NO: 5) were considerably lower to that of MHOM/IN/KE16/1998 (SEQ ID NO: 6) for the same group of samples. The preliminary study carried out with sera from 72 Endemic and 80 non-endemic healthy controls, 92 Tuberculosis positive, 32 HIV positive, 31 HCV positive and 27 HBsAg positive individuals. These ELISA results showed 100% specificity with no cross reactivity. However, ELISA carried out for confirmed VL (N=10) and PKDL (N=10) patient's sera with rK39 antigen from *L. chagasi* (Burns *et al.,* 1993), *L. donovani* antigen from the strains MHOM/IN/DD8 (SEQ ID NO: 5) and MHOM/IN/KE16/1998 (SEQ ID NO: 6) show high antibody titer for all three antigens at 50ng/ well concentration. The data show that, these two well-characterized antigens from the Indian strains are highly specific and sensitive for the kala-azar diagnosis in India. In a study conducted with more samples it was found that considerable number of cases that could not be diagnosed by rK39 of *L. chagasi* was detected by these two recombinant polypeptides from *L. donovani.* This reveals that, the newly isolated recombinant polypeptides from Indian strains of *L. donovani* are more specific for the diagnosis of VL and PKDL in India thus they can be used for diagnosis of this disease extensively.

However, only the data pertaining to 10 positive samples and other control samples are presented in figure 15 and figure 16. The figure 15 depicts the mean titer value of different samples subjected to ELISA using purified antigen of MHOM/IN/DD8 origin and the figure 16 show the mean titer value of different set of samples for the purified antigen of MHOM/IN/KE16/1998 origin.

EXAMPLE 4

This example teaches obtaining antibody against the polypeptides SEQ ID NO: 5 or SEQ ID NO; 6 from an animal and its use in the detection of *Leishmania* antibody as below;

1. Purified recombinant polypeptides from the group containing SEQ ID NO: 5 and SEQ ID NO: 6 were used for immunization in rabbits. 100 micro gram of the polypeptide was emulsified with equal volume of Freund's complete adjuvant (Sigma, USA).
2. The mixture was injected intradermally at multiple sites.
3. Three booster doses of the same amount of antigen emulsified with incomplete Freund's adjuvant were given at 30-day intervals. Prior to the first immunization, preimmune serum was collected and tested on western blots.
4. Blood was collected aseptically from the immunized animals 12 days after the last booster, sera were separated and the anti –antibody present in the sera was affinity purified and stored at 4^0C until use.
5. The antibody was first diluted in 0.1M Bicarbonate buffer, pH 9.2 and then 200 µl are added to each well of the microtiter plate.
6. The antibody coated plate was covered with Parafin and incubated in the cold room overnight in a moist box containing a wet paper towel or at room temperature and humidity for two hours.
7. The plate is emptied and the unoccupied sites are blocked with 100 µl of blocking buffer containing 100 mM phosphate buffer, pH 7.2, 1% BSA, 0.5% Tween-20 and 0.02% Thimerosol for 30 min at room temperature.
8. The plate is emptied and washed three times with wash buffer (100 mM phosphate buffer, 150 mM NaCl, 0.2% BSA and 0.05% Tween 20).
9. The antigen solution is first diluted in antigen buffer (100 mM phosphate buffer, 150mM NaCl) and then added to the plate in a volume of 50 µl per well. The plate is incubated at room temperature for 45 min to one hour.
10. The plate is emptied again and washed three times with wash buffer.
11. The enzyme-labeled antibody against antigen is diluted appropriately in 0.1M Bicarbonate buffer, pH 9.2 and then 50 µl is added to each well and incubated at room temperature for 30 min.
12. The plate is emptied again and washed three times with wash buffer.

13. The color development system is added and the color intensities are measured.

ADVANTAGES:

- The characterization of the kinesin related antigen at the sequence level has revealed the similarities and differences for the kinesin antigen between two species *L. donovani* and *L. chagasi*.
- It is clear from this invention that the immunodominant repeat of kinesin related antigen isolated from Indian strains is different from the earlier reported sequences of other *Leishmania* species.
- This study revealed that the Indian strains are quite different from the earlier reported strains and species from other geographical regions.
- The study also revealed that amino acid sequence variation between the repeat units of the Indian strains is very high than for previously reported sequences.
- This invention has led to the isolation of polypeptide, which is different in sequence from that of earlier reported polypeptide sequence.
- This invention has led to the development of a method for detection of antileishmanial antibodies based on the newly found antigens from Indian isolates of *L. donovani*.
- This invention has led to the leishmaniasis detection kit for the detection of VL due to species of *L. donovani* of India and other similar species which would have been missed by K39 polypeptide

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